

Feeding salicylates containing willow leaves to cattle modulates urea metabolism and mitigates urine-derived ammonia and nitrous oxide emissions from soil

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ABSTRACT

Ruminants on grazing lands have a great impact on ammonia (NH₃) and nitrous oxide (N₂O) emissions released from livestock production. Willow leaves are an established supplement in ruminant nutrition and are rich in salicylates and tannins, which may have a mitigating effect on NH₃ and N₂O emissions. We hypothesised that willow leaf supplementation in cattle nutrition affects nitrogen (N) and urea metabolism and mitigates urinary NH₃ and N₂O emissions from soil. Eight weaned Holstein bull calves were kept on pasture and supplemented with willow leaves or alfalfa hay in a crossover design. In a respiration chamber, feed intake, faeces and urine excretions were recorded and analysed for total N and N-metabolites. Urea-N recycling was measured by the intravenous administration of a ¹³C urea tracer and a series of blood sampling. Cattle urine and artificial mimics supplemented with different salicylates were incubated with standard soil to measure NH₃ and N₂O and the N and O isotopic signatures. Despite a decline in urea turnover and N digestibility in rations supplemented with willow leaves, the leaves had no effect on microbial protein synthesis or the growth rate. Urine excretions with reduced urea but increased hippuric acid, phenolic acids, and salicylate concentrations in cattle fed willow leaves mainly inhibited bacterial denitrification processes involved in N₂O release from soil and mitigated NH₃ and N₂O emissions by 14 and 81 %, respectively. The results highlight intrinsic and extrinsic mechanisms that define both the nutritional significance and emission mitigation potential of supplementing cattle in pastures with willow leaves.

1. Introduction

Agriculture is the main emitter of gaseous nitrogen (N) compounds, making up more than 80 % of all global ammonia (NH₃) (Van Damme et al., 2021) and 81 % of total nitrous oxide (N₂O) emissions (IPCC, 2019). Although emitted NH₃ is returned to the Earth's surface within a short time, amplifying soil acidification, ecosystem eutrophication, and species community changes (García-Gómez et al., 2014), atmospheric N₂O possessing an atmospheric lifetime of about 150 years acts as a potent greenhouse gas and dominant ozone-depleting substance (Ravishankara et al., 2009). Manure excretions from grazing livestock in

pastures and rangelands account for almost 26 % of these anthropogenic N emissions from agriculture, currently amounting to up to 5.7 Mt NH₃-N (Beusen et al., 2008) and 1.9 Mt N₂O-N (FAO, 2021) per year. Grassland ecosystems worldwide are predominantly grazed by ruminant species, including cattle, sheep, and goats, who may utilise less than 10–40 % of the ingested N, whereas 60 % to more than 90 % is excreted as dung and urine (Calsamiglia et al., 2010; Carswell et al., 2019). Because N compounds in urine are reactive, water-soluble molecules, the fraction of N lost as N₂O and NH₃ in urine is greater than that in faeces (Cai et al., 2017).

The main N compound in urine is urea, which is rapidly hydrolysed

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in the soil to ammonium (NH_4^+) that can be directly volatilised as NH_3 . Ammonium can also be converted to nitrite (NO_2^-) and then to nitrate (NO_3^-) by nitrifying bacteria. As a by-product of nitrification, but also during the reduction of NO_2^- and NO_3^- to N_2 in nitrifier denitrification or denitrification, N_2O is formed (Cai et al., 2017). The magnitude of NH_3 and N_2O emissions depends on the soil microclimate, microbial activity, botanical composition and urinary urea excretions (Rivera and Chara, 2021).

In grazing ruminants, there is a great potential to mitigate NH_3 and N_2O emissions by modulating the urine composition through dietary means: (a) by reducing the N intake and elevating the level of the rumino-hepatic urea N cycling, e.g., by managing the intensity and timing of grazing (Rivera and Chara, 2021), and (b) by including tannin-rich plants in the diet. Tannin intake inhibits protein digestion in the rumen, thereby providing less NH_3 for ruminal microbial protein synthesis, urea synthesis in the liver, rumino-hepatic urea-N cycling, and urinary urea excretion. Furthermore, absorbed tannins excreted with urine inhibit the conversion of urinary urea to NH_3 in soil (Powell et al., 2011) and facilitate urinary hippuric acid excretion (van Cleef et al., 2022), which in turn inhibits N_2O formation from excreta deposited on soil (van Cleef et al., 2022). Thus far, the importance of further secondary plant compounds capable of reducing urine-derived NH_3 and N_2O emissions needs to be determined.

Leaves from willow trees (*Salix* spp.) are rich in salicylates, and although they contain tannins, they are used as a nutritious feed supplement for cattle and sheep grazing on sparse pastures in New Zealand (Moore et al., 2003) or in montane riparian ecosystems of the Northern Hemisphere, the latter of which is characterised by short growing seasons and limited cultivation opportunities (Phillips et al., 1999). The predominant salicylates in willow leaves are tremulacin, salicortin, and salicin (Schmid et al., 2001), which are cleaved by gastrointestinal β -glucosidases after ingestion, forming salicylic alcohol; the latter is then further oxidised to salicylic acid (Buss, 2005). Salicylic acid is mainly excreted in urine but is also partially metabolised in the liver to salicyluric acid and gentisic acid before being excreted in these forms (Schmid et al., 2001). Recently, *ab initio* studies based on Raman spectroscopic investigations have identified the formation of strong molecular bonds between urea and salicylic acid that can prevent urea hydrolysis and associated NH_3 emissions (Silva et al., 2020). In addition to its inhibitory effect on bacterial urease activity (Mao et al., 2009), salicylic acid inhibits ruminal proteolysis (Kingston-Smith et al., 2012). However, its impact on ruminal NH_3 formation, hepatic urea production, urinary urea excretion, and related NH_3 and N_2O emissions from soil remains unexplored. We hypothesised that supplementing cattle diets with willow leaves containing tannins and salicylates would reduce ruminal NH_3 release, hepatic urea production and alter the urinary urea and salicylate concentrations, resulting in less volatile N emissions from pasture soils. This hypothesis was tested in a novel interdisciplinary research approach combining animal and soil sciences, which will reveal intrinsic and extrinsic mechanisms defining the nutritional and emission mitigation potential of willow leaves as a supplement in cattle nutrition.

2. Materials and methods

All experimental procedures were approved by the Office of Agriculture, Food Security and Fishery Mecklenburg-Western Pomerania, Germany (No. 7221.3-1-004/22). The animal experiment was conducted between May and August 2022 at the Research Institute for Farm Animal Biology (FBN), Germany.

2.1. Animals and treatments

Eight newborn German Holstein bull calves entered the trial in 2 blocks (each $n = 4$). Calves were raised under comparable conditions and were weaned at 12 weeks of age. After weaning, the calves were adapted to pasture feeding for two weeks (Experimental Design:

Supplemental Figure S1). The adaptation period involved the frequent quantification of body weight (BW) and the adaptation to the institute's respiration chambers. During the following experiment, calves on pasture were supplemented with either concentrates and willow leaves (*Salix* spp., SAL; Alfred Galke GmbH) or concentrates and alfalfa hay (CON) in a crossover design (Table 1, Supplemental Table S1), including two feeding and sampling periods per block. The dry matter intake was estimated to be 2.4 % of BW, and BW was recorded twice weekly. Supplements amounted to 457 ± 36 g/kg dry matter (DM) intake and fresh grass intake on pasture or grass clippings comprised the remaining 543 ± 36 g/kg DM intake (Table 1). Both diets were formulated as isoenergetic (9.7 ± 0.1 MJ/kg DM) and isonitrogenous (134.5 ± 13.0 g/kg DM).

2.2. Experimental schedule

On each diet, calves were adapted to the supplements for two weeks (adaptation or washout period). Both SAL and CON were mixed with water and provided in individual troughs twice daily. Throughout the

Table 1

Nutrients in diets containing willow leaves (SAL) or alfalfa hay (CON). Means $\pm$ SD.

Component ^a	SAL	CON
Ingredients, g/kg of DM		
Fresh grass (clippings) ^b	541 $\pm$ 43	542 $\pm$ 27
Concentrate pellets ^c	160 $\pm$ 15	152 $\pm$ 10
Wheat meal	90 $\pm$ 8	130 $\pm$ 8
Willow leaves	209 $\pm$ 20	—
Alfalfa hay	—	176 $\pm$ 9
Nutrients, g/kg of DM		
Crude ash	101 $\pm$ 19	101 $\pm$ 20
Crude protein	134.1 $\pm$ 12.6	134.9 $\pm$ 13.4
Crude fat	27.6 $\pm$ 1.7	27.0 $\pm$ 1.7
Crude fibre	208 $\pm$ 11	238 $\pm$ 10
ADF	238 $\pm$ 14	263 $\pm$ 11
aNDF	425 $\pm$ 19	449 $\pm$ 16
ADL	49 $\pm$ 2	46 $\pm$ 2
Starch	92 $\pm$ 8	116 $\pm$ 9
Sugar	67 $\pm$ 16	60 $\pm$ 16
ME, MJ/kg DM ^d	9.7 $\pm$ 0.1	9.7 $\pm$ 0.1
Plant secondary compounds, g/kg of DM		
Phenolic acids (tannic acid-equ.)	18.6 $\pm$ 1.6	5.2 $\pm$ 0.7
Condensed tannins (procyanidin-equ.)	26.7 $\pm$ 2.5	—
Flavonoids (quercetin-equ.)	0.79 $\pm$ 0.06	0.56 $\pm$ 0.04
Salicylates	3.17 $\pm$ 0.30	0.01 $\pm$ 0.01
Salicin	0.23 $\pm$ 0.02	0.01 $\pm$ 0.01
2'-O-Acetylsalicin	0.33 $\pm$ 0.03	—
Salicortin	0.53 $\pm$ 0.05	—
2'-O-Acetylsalicortin	0.55 $\pm$ 0.05	—
3'-O-Acetylsalicortin	0.53 $\pm$ 0.05	—
Tremulacin	0.86 $\pm$ 0.08	—
Lasiandrin	0.14 $\pm$ 0.01	—

^a ADF = acid detergent fibre; ADL = acid detergent lignin; aNDF = neutral detergent fibre after amylase treatment, equ. = equivalent; DM = dry matter.

^b Calculated by average daily intake of fresh grass clippings during experimental week and nutrient analyses within proportionally pooled fresh grass samples per period sampling.

^c BERGIN Kälberpellet (Bergophor Futtermittelfabrik Dr. Berger GmbH & Co. KG, Kulmbach, Germany): extracted soy meal from peeled and steam-heated beans, dried sugar beet pulp, wheat semolina bran, pulped cord, products and by-products from bakery and pasta industry, apple molasses, apple pomace, pulped linseed, pulped carob, $\text{Ca}(\text{H}_2\text{PO}_4)_2$, CaCO_3 , wheat bran, protein rich by-product from fungal solids fermentation. Additives per kg original substance: 16,000 IU vitamin A (rumen-protected), 4000 IU vitamin D₃, 200 mg vitamin E, 300 mg vitamin C, 4 mg vitamin K₃, 15 mg vitamin B₁, 8 mg vitamin B₂, 10 mg vitamin B₆, 50 μg vitamin B₁₂, 50 μg vitamin B₁₂ (rumen-protected), 20 mg Ca-D-Pantothenate, 40 mg niacin amide, 4 mg folic acid, 0.3 mg biotin, 150 mg Fe, 12 mg Cu, 60 mg Zn, 50 mg Mn, 1 mg I, 0.4 mg Se. Composition: 1.2 % Ca, 0.6 % P, 0.3 % Na.

^d Metabolisable energy content was calculated according to GfE (2001).

adaptation or washout period, calves had free access to the barn, pasture, grass clippings, water and lick stone.

After the adaptation or washout procedure, calves were transported to the institute's experimental facility and housed in tie-stalls for five days. On days 1 and 2, 100 % of daily supplements and 30 % of daily grass clippings were fed at 0800 h and further grass clippings were fed *ad libitum* at 1900h. On days 3 and 4, calves were fed in 3-h intervals between 0800 and 1700 h and received 100 % of daily SAL or CON intake and 50 % of daily grass clippings intake in four equal portions. At 2000 h, the calves received further grass clippings for *ad libitum* intake. On day 5, calves received 100 % of daily SAL or CON intake and grass clippings for *ad libitum* intake at 0800 h. Feed and water intake were quantified every 24 h.

On day 1, rumen fluid was sampled via oral tubing at 1000 h. Subsequently, calves were equipped with a jugular venous catheter flushed with 0.9 % NaCl solution. At 1200 h, the calves were weighed and individually transferred into a respiration chamber. Calves were equipped with a urinal connected to a bucket and a vacuum pump. Urine released within the first 24 h was collected to determine the urine density. On days 2–5, the bucket was pre-filled with 365 g of 25 % sulphuric acid to ensure pH < 3.0. Urine and faeces were collected, quantified and sampled every 24 h.

Respiratory measurements were performed between days 1 (1200 h) and 2 (1900 h), with a 7-h equilibration period and a 24-h measurement period. Measurements were conducted at 20 °C, 70 % humidity, and an airflow of 15 m³/h. Gas samples were drawn in 6-min intervals by a membrane pump to analyse the concentrations of oxygen (O₂), carbon dioxide (CO₂), methane (CH₄) and NH₃ (SIDOR and GMS800; SICK MAIHAK GmbH). Heat production (HP) was calculated by daily gas emissions relative to the metabolic BW (mBW) as follows (Brouwer, 1965): $HP/mBW (kJ/kg^{0.75}) = [16.18 \times O_2 (L/d) + 5.02 \times CO_2 (L/d) - 2.17 \times CH_4 (L/d) - 5.99 \times N_{Urine} (g/d)]/mBW (kg^{0.75})$. Urinary N excretions (N_{Urine}) were analysed in pooled acidified urine samples, as outlined below. The energy balance was calculated as the difference between the metabolisable energy intake and HP.

A ¹³C urea tracer study was conducted on days 3 (*n* = 4, group A) and 4 (*n* = 4, group B) during the interval feeding regime as described (Müller et al., 2021). The natural abundance of ¹³C urea was determined in two blood samples taken 5 and 10 min before intravenous administration of ¹³C urea via a jugular venous catheter. The ¹³C urea tracer (¹³C urea; ≥ 99 atom%; Sigma-Aldrich) was administered as a bolus (3.5 mg/kg BW) and immediately flushed with 20 mL of saline solution. Blood samples were taken 5, 10, 20, 30, 60, 120, 180, 240, 360, 480, 600 and 1320 min after bolus administration in 9-mL EDTA tubes (Sarstedt). An aliquot was centrifuged at 1345 ×g (20 min, 4 °C). Plasma and whole blood aliquots were stored at –20 °C until further analysis. On day 5 at 1200 h, calves were weighed and transferred back to the pasture and barn areas to receive the second supplement (SAL or CON) for two weeks (washout period), followed by a second experimental sampling period of five days.

2.3. Feed nutrients

Willow leaves, alfalfa hay, wheat meal and concentrate pellets were fed from one lot throughout the experiment and sampled once, while green clippings were sampled per batch. The dry matter content was determined by air-drying at 60 °C for 1 day, followed by drying at 103 °C for 4 h. Subsequently, green clipping samples were proportionally pooled according to the average week's intake. Except for acid detergent fibre (ADF) and acid detergent lignin (ADL), the nutrient content of concentrate pellets was provided by the manufacturer. Nutrients of the remaining feedstuffs (willow leaves, alfalfa hay, wheat meal, pooled green clippings) and ADF and ADL in concentrate pellets were analysed in dried and milled (0.7 mm) feed samples as defined previously VDLUFA (1997). The metabolisable energy content was calculated in wheat meal according to GfE (2001).

2.4. Phenolic compounds and benzoic acid in feed (VDLUFA, 1997) and urine

To extract phenolic compounds, dried and milled feed samples (50 mg) or non-acidified urine (5 mL) were mixed with 10 mL or 5 mL of acetone (50 %, v/v), respectively. After shaking for 24 h, samples were centrifuged (1245 ×g, 15 min, 4 °C) to obtain the supernatant. The concentrations of phenolic acids and tannins in feed and urine extracts were determined in triplicate as tannic acid equivalents using the Folin–Ciocalteu reagent before and after treatment of the extracts with polyvinylpyrrolidone (Sigma-Aldrich) (FAO and IAEA, 2000). Condensed tannins were determined as procyanidin B2 equivalents according to the HCl–butanol–acetone assay (Shay et al., 2017). Briefly, 100 µL of extract was mixed with 600 µL butanol–HCl reagent and 20 µL Ferric reagent (Fe-III-chloride hexahydrate) in triplicate. After incubation at 70 °C for 4 h, the absorption was determined at λ = 550 nm. To extract flavonoids, dried and milled feed samples (1 g) was mixed with 20 mL of ethanol (75 %, v/v), incubated at 60 °C for 30 min and filtered three times. A 200 µL sample of the extract was mixed with 800 µL of ethanol (70 %, v/v) and incubated for 30 min. The flavonoid concentration was determined in duplicate as quercetin equivalents at λ = 410 nm.

Bioactive salicylates identified in willow bark (Antoniadou et al., 2021) have been measured in all feed samples by targeted UHPLC-MS/MS_{MRM}. Powdered feed material (5 mg) was extracted following the literature protocol (Antoniadou et al., 2025). The extracts were analysed by targeted UHPLC-MS/MS_{MRM} using a QTRAP 6500 mass spectrometer (Sciex) coupled to a Nexera X2 UHPLC system (Shimadzu) (Antoniadou et al., 2025). In addition, benzoic acid was analysed using the following MRM transitions (Q1/Q3, Da): 120.90 → 76.80, 120.90 → 119.00 and 120.90 → 114.00 (Supplemental Table S2). All quantitative data were obtained in triplicate.

The urinary concentrations of benzoic acid, salicylic acid, salicyluric acid and salicyl alcohol were determined in tenfold diluted non-acidified urine samples by HPLC (1200/1260 infinity Series; Agilent Technologies). Samples were separated on a 250 × 4.6 mm Synergi 4 µm Hydro-RP 80 Å column protected by a 4 × 3 mm pre-column (Phenomenex). Phosphate buffer (20 mM, pH 6.5) was used as an eluent at a flow rate of 1 mL/min and with a gradient of acetonitrile ranging from 0 % to 10 % for 5–25 min. Finally, salicylates were detected at λ = 210 nm and benzoic acid at λ = 230 nm.

2.5. Total N and fibre analyses

Faecal samples were dried at 60 °C to determine the dry matter content. Dried faecal samples, collected between days 1 and 5, and acidified urine samples, collected between days 2 and 5, were pooled separately according to the amount excreted within 24 h for each animal. The N and amylase-treated neutral detergent fibre (aNDF) contents were analysed in proportionally pooled faecal samples as described in Section 2.3.1 and urinary N concentrations in proportionally pooled urine samples according to Dumas (VDLUFA, 1997). The N balance was calculated as the difference between the average N excretion and the average N intake on sampling days (days 1–5). The N use efficiency was defined as the N balance relative to the average N uptake. The apparent digestibility (AD) of N, aNDF and DM was calculated as the ratio between the average retained (ingested amount per day – excreted amounts faeces per day) and average ingested N, aNDF and DM per day.

2.6. N metabolites in plasma and urine

Plasma samples obtained 240, 360 and 1320 min after ¹³C urea administration were equally and separately pooled for each animal. Non-urea N concentrations were determined in pooled plasma samples and in tenfold diluted non-acidified urine samples via HPLC, as described in section 2.3.2. Allantoin and creatine were detected at λ

= 210 nm and the remaining metabolites at $\lambda = 230$ nm. Urea was analysed in 50-fold diluted proportionally pooled acidified urine samples by using HPLC (Müller et al., 2021) and detected using the refractive index. The urea concentration in pooled plasma samples was measured spectrophotometrically using a clinical chemistry analyser (ABX Pentra C400; HORIBA) with an A11A01–641 kit (HORIBA).

2.7. Rumen fluid pH, NH_3 and hydrogen (H_2) concentrations

Immediately after sampling, the rumen fluid was sieved and the pH of the rumen fluid was determined. The ammonia concentration was analysed in sieved rumen fluid using Conway microdiffusion analysis (Kenten, 1956). The H_2 concentration was analysed in sieved rumen fluid, as described (Wang et al., 2014). The hydrogen (H_2) concentration in the gaseous phase (C_{gH_2}) was immediately determined on an electrochemical H_2 analyser (Lactotest 202 Xtend; Medical Electronic Construction). The H_2 concentration in the sieved rumen fluid (C_{dH_2}) was calculated according to $\text{C}_{\text{dH}_2} = \text{C}_{\text{gH}_2}/22.4 \times (\alpha + \text{V}_{\text{g}}/\text{V}_{\text{rf}})$ (Wang et al., 2014), in which the volume of the gas phase (V_{g}) is divided by the volume of rumen fluid (V_{rf}).

2.8. Plasma ^{13}C urea and whole blood $^{13}\text{CO}_2$ enrichments

The ^{13}C urea enrichment analyses in plasma samples were performed as described (Müller et al., 2021). Briefly, after deproteinisation with acetonitrile and incubation with *N*-methyl-*N*-(*t*-butyldimethylsilyl)-tri-fluoroacetamide (MTBSTFA) and pyridine, the intensities of the resulting ^{12}C and ^{13}C *t*-butyldimethylsilyl derivatives were detected on a gas chromatograph-mass spectrometer (GC-MS, QP 2010, coupled with GC 2010, AOC-20i; Shimadzu) at m/z 231 and 232, respectively. The ^{13}C abundances were converted into enrichments in (E) in mol% excess (MPE) considering the basal values (see above). A two-exponential curve fitting (enrichment as a function of time (t) = $a \times e^{(-b \times t)} + c \times e^{(-d \times t)}$) was applied using TableCurve 2D (ver.5.0.1; Systat), and the area under the enrichment-time-curve ($\text{AUC} = a/b + c/d$) was calculated. Subsequently, the whole-body ^{13}C urea turnover rate, the urea entry rate (UER), the urea pool size (Q) and the fractional disappearance rate of ^{13}C urea (K_{Urea}) were calculated (Wolfe and Chinkes, 2005) with regard to the tracer dosage and BW. The urea space was calculated as the urea pool size related to the plasma urea concentration in mg/L. Finally, the urea entrance rate into the gastrointestinal tract (GER) was calculated as follows: $\text{GER} (\text{g urea-N/d}) = \text{UER} (\text{g urea-N/d}) - \text{urinary urea N} (\text{g/d})$ (Spek et al., 2013; Müller et al., 2021). Accordingly, rumino-hepatic urea-N was recycled as $\text{GER}/\text{UER} \times 100 \%$. The $^{13}\text{CO}_2$ enrichment in whole blood samples and the microbial urea hydrolysis rate were determined as described (Müller et al., 2021). Briefly, a 1-mL sample of whole blood was treated with 1 mL of 10 % lactic acid, and the ratio between $^{13}\text{CO}_2$ and $^{12}\text{CO}_2$ was determined on an isotope ratio mass spectrometer (IRMS, DELTAplus XL; Thermo Quest) coupled with a GasBench II (Finnigan). After conversion into atom % excess (APE), the enrichment time curve was submitted to best curve fitting (TableCurve 2D) to obtain the AUC and average ^{13}C enrichment in CO_2 per day. The urea hydrolysis rate was calculated from the CO_2 respiratory measurements and the $^{13}\text{CO}_2$ enrichments in whole blood (Slater et al., 2004; Müller et al., 2021).

2.9. Soil incubation and associated N_2O and NH_3 gaseous measurements

In the first incubation experiment, non-acidified urine samples were pooled separately for the CON ($n = 8$) and SAL ($n = 8$) diets. Dried standard soil (LUF, Speyer) was mixed with demineralised water to reach 40 % of the water-holding capacity (WHC) and 200 g of soil per incubation jar (850 mL). After 2 days of incubation at room temperature, the prepared soil was mixed with 30 g of either the pooled urine samples (CON: $n = 5$; SAL: $n = 5$) or water ($n = 2$), reaching 80 % WHC. In a second incubation experiment, artificial urine (AU) was mixed

according to the average urine composition on the CON diet (7.08 g/L urea, 0.15 g/L uric acid, 1.09 g/L allantoin, 0.79 g/L creatinine and 0.78 g/L creatine) with distilled water. For the treatments, this AU was supplemented with either salicylic acid (53.94 mg/L), salicyluric acid (101.36 mg/L), salicyl alcohol (292.32 mg/L), with all three of these salicylates, hippuric acid (10.08 mg/L), or hippuric acid and salicylates in concentrations found in the urine of SAL calves. Prepared soil (85 g, WHC 40 %) was incubated for 2 days in incubation jars (500 mL) and mixed with 12.02 g of AU alone ($n = 5$), AU with the additives (each, $n = 5$), or water ($n = 2$) to reach 80 % WHC.

Absorbent filter paper (Whatman No. 1823–025) soaked with KHSO_4 (2.5 M) was placed in the lid of each jar before closing, and the jars were incubated at room temperature. After 1, 3, 6, 24 and 48 h of incubation, 20-mL gas samples were taken from the headspace with a syringe and analysed for N_2O and associated isotopic signatures using a trace gas pre-concentrator coupled to an isotope ratio mass spectrometer (IRMS, IsoPrime 100; Elementar) (Berendt et al., 2023). The detection limits of the lowest and highest gaseous N_2O -N content were 0.11 and 88.75 ppm, respectively. The external precision of ^{15}N in N_2O was 0.14 ‰, on average.

At 6, 24 and 48 h, the acid-soaked filter papers were exchanged for new ones, and specific time points of opening the chambers were considered within the N_2O flux calculations. After 48 h of incubation, 20 g of soil was sampled from each jar to extract NH_4^+ and NO_3^- using the KCl extraction method (Brooks et al., 1989). For isotopic analysis, NH_4^+ and NO_3^- were transformed to NH_3 and caught on absorbent filter paper soaked with KHSO_4 using MgO and Devarda's Alloy, respectively (Brooks et al., 1989; Wrage et al., 2005). Absorbent filter papers were obtained from the incubation approaches, and the NH_4^+ and NO_3^- extracts were weighed into tin capsules. Their N content and isotopic signature were measured on an elemental analyser (vario PYRO cube; Elementar) coupled with IRMS. As internal standards for the determination of NH_4^+ and NO_3^- contents and isotopic signatures in soil samples, we used sulphanilamide and wheat flour calibrated against IAEA-600.

Isotopic signatures $^{15}\text{N}/^{14}\text{N}$ and $^{18}\text{O}/^{16}\text{O}$ were reported relative to the international standards AIR- N_2 and Vienna Standard Mean Ocean Water (VSMOW). The following equations were used to calculate the isotope ratios (δ) (Coplen, 2011): $\delta^{15}\text{N} [\text{‰}] = \left(\frac{(^{15}\text{N}/^{14}\text{N})_{\text{Sample}}}{(^{15}\text{N}/^{14}\text{N})_{\text{Standard}}} - 1 \right) \times 1000$; $\delta^{18}\text{O} [\text{‰}] = \left(\frac{(^{18}\text{O}/^{16}\text{O})_{\text{Sample}}}{(^{18}\text{O}/^{16}\text{O})_{\text{Standard}}} - 1 \right) \times 1000$; site preference (SP) = $\delta^{15}\text{N}_{\text{N}_2\text{O}} - \delta^{15}\text{N}_{\text{N}_2\text{O}}$.

2.10. Statistics and upscaling

Power analysis to determine a sufficient sample size was performed using the pwr.t.test function included in the R package 'pwr' (Champely et al., 2020). Testing 8 animals in a crossover design with a specific likelihood (statistical power, $1 - \beta$ / Type II error) of 0.80 and an α -level of 0.05 reached a Cohen's $d = 1.2$. One animal on the CON diet suffered from fever (rectal temperature 39.9 °C for 1 day) during the adaptation period 5 days prior to sampling. However, determining the interquartile range of each parameter did not reveal any outliers; thus, data from all animals were included in the statistical evaluation.

Animal-related data were statistically analysed by repeated measurement analyses of variance using the MIXED procedure in SAS (version 9.4, SAS Institute Inc.). The model considered the diet (SAL versus CON), feeding and sampling period (1–4) and their interaction as fixed effects (Supplemental Table S3). The REPEATED statement was considered, with repeated measurements on the same calf defined as SUBJECT = animal option and an unstructured covariance structure. Degrees of freedom approximation was performed by the Kenward-Roger method and a two-sided confidence interval of 95 % was applied. The least squares means (LSM) and standard error (SE) were computed for each fixed effect, and pairwise multiple comparison was tested using the Tukey–Kramer test (Supplemental Table S3). The statistical model was designed as follows:

$$y_{ijk} = \mu + a_i + \beta_j + (a\beta)_{ij} + e_{ijk}$$

y_{ijk} : response variable, μ : average test score, a_i : fixed effect of diet on level i , β_j : fixed effect of feeding and sampling period on level j , $(a\beta)_{ij}$: two-times interaction between diet on level i and feeding and sampling period on level j , e_{ijk} : independent $N(0; \sigma^2_{ijk})$ -distributed experimental error term.

The order of treatment was not included as a fixed effect due to model overfitting. However, including order as a fixed effect revealed no effects on tested parameters despite overfitting. Significance was defined at a P -value < 0.05 and tendencies were defined at $0.05 < P < 0.10$. The results are presented as $LSM \pm \text{mean}$.

Average and temporal N_2O and NH_3 emissions, isotopic signatures and NH_4^+ and NO_3^- soil concentrations were analysed using the TTEST procedure in SAS, considering the average and temporal pattern of each incubation vessel. We compared the pattern between soils incubated with pooled urine derived from CON- and SAL-fed calves, between soils incubated with AU alone, and soils incubated with AU mixed with additives as independent classifications. A two-sided confidence interval of 95 % was applied. The normal distributions of the dependent variables were tested using the UNIVARIATE procedure. If metric variables were not normally distributed, a Johnson transformation (Hemmerich, 2016) was applied. Equality of variance was tested using the folded F-statistics included in the TTEST procedure. If the assumption of equality was reasonable, the pooled t -test was evaluated. If the assumption of equality was not reasonable, the alternative Satterthwaite adjustment for degrees of freedom was applied (Supplemental Table S3). Results were considered significant at $P < 0.05$ and tendencies between $0.05 < P < 0.10$. The results are presented as the mean $\pm$ SEM (Supplemental Table S3).

A Markov-Chain Monte Carlo model was implemented in Fractionation and Mixing Evaluation (FRAME) (Lewicki et al., 2022) to estimate the fractional contributions of individual sources and processes involved in N_2O release. This 3D modelling approach considered the 48-h averaged isotopic signatures of the isotope ratio of ^{15}N and ^{14}N in N_2O ($\delta^{15}N_{N_2O}^{\text{bulk}}$), the isotope ratio of ^{18}O and ^{16}O in N_2O ($\delta^{18}O_{N_2O}$), and the site preference of ^{15}N towards the α or β N position in N_2O ($\delta^{15}N_{N_2O}^{\text{SP}}$) determined during the incubation experiments with AU. Moreover, we included the stable isotope composition of the pathways: bacterial denitrification (bD), nitrifier denitrification (nD), fungal denitrification (fD), nitrification (Ni) and the reduction fractionation factor as auxiliary parameters (Lewicka-Szczepak et al., 2020). The substrate-corrected $\delta^{18}O$ for each pathway was obtained by applying the substrate isotope value $\delta^{18}O_{H_2O}$ (Lewicka-Szczepak et al., 2020). We added the average $\delta^{15}N$ of NO_3^- to obtain the substrate-corrected $\delta^{15}N$ of bD and fD and the average $\delta^{15}N$ of NH_4^+ to obtain the substrate-corrected $\delta^{15}N$ of nD and Ni (Lewicka-Szczepak et al., 2020) (Supplemental Table S4). The mean residual unreduced N_2O -N fraction (r_{N_2O}) determined in FRAME was further used to estimate the release of N_2 -N since $r_{N_2O} = \gamma_{N_2O} / (\gamma_{N_2O} + \gamma_{N_2})$ (γ : mole fraction; Lewicki et al. 2022), as follows:

$$r_{N_2} = 1 - \text{average } r_{N_2O};$$

$$N_2\text{-N } (\mu\text{g} \times \text{h}^{-1} \times \text{kg}^{-1} \text{ soil}) = N_2O\text{-N } (\mu\text{g} \times \text{h}^{-1} \times \text{kg}^{-1} \text{ soil}) / \text{average } r_{N_2O} \times r_{N_2};$$

$$N_2\text{-N } (\% \text{ of urine-N} \times \text{d}^{-1}) = N_2O\text{-N } (\% \text{ of urine-N} \times \text{d}^{-1}) / \text{average } r_{N_2O} \times r_{N_2}.$$

The N_2O and NH_3 emission mitigation potential of willow leaves supplemented to cattle on pasture was scaled up to a global level using emission data from the Food and Agricultural Organisation (FAO (2023)). We considered data on N_2O emissions and the N content derived from manure left on pasture by non-dairy cattle in 2021 and in countries with comparable climatic conditions and willow species (Argus, 2010; Durrant et al., 2016): Europe (except Iceland, Faroe Islands and Russia), Canada, USA and New Zealand. Approximately 91.1 % of N_2O -N emissions from cattle manure left on pasture are derived from urine patches

(Voglmeier et al., 2019). Total N volatilised as NH_3 accounts for 22.4 % of N excreted by cattle on pasture, and urine patches account for 88.6 % of these NH_3 emissions (Laubach et al., 2013). Considering the latter and the respective N_2O and NH_3 emission reduction determined in the present study, the absolute N_2O and NH_3 reduction potential upon willow leaf supplementation to non-dairy cattle on pasture was calculated for each country.

3. Results

3.1. Feed intake, methane and N excretions

Daily consumption of water and feed DM was comparable between feeding groups (Supplemental Table S5). Further, body condition, BW, average daily gain, energy balance and energy use efficiency did not differ between the groups. Respiratory measurements revealed that the NH_3 , CO_2 and CH_4 emissions relative to NDF intake were comparable in both diets. However, calves fed the SAL diet released 8 % less CH_4 when normalised to the metabolic BW ($P = 0.010$) than calves fed the CON diet. Analyses of N excretions showed a trend towards a 22 % increase in faecal N excretions in SAL-fed calves compared to CON-fed calves (49.6 vs. 40.7 g/d, $P = 0.082$), but at a comparable level of urinary N excretion (Table 2). The elevated faecal N excretion on the SAL diet was paralleled by a 15 % decline in N balance (30.9 vs. 36.5 g/d, $P = 0.061$) and an 8.7 % reduction in AD of N (52.7 vs. 61.4 %, $P = 0.003$).

3.2. Plasma N metabolites, urea flow and urea recycling

The plasma concentrations of allantoin ($P = 0.015$) and creatine ($P = 0.037$) increased by 8 and 7 %, respectively, in calves fed the SAL diet, while the plasma hippuric acid concentration was almost twice as high in calves fed the SAL diet as in those fed the CON diet (142 vs. 79 μM , $P = 0.005$) (Supplemental Table S6). However, we found that the plasma uric acid ($P = 0.015$) and urea ($P = 0.028$) concentrations diminished in SAL-fed calves by 10 and 14 %, respectively, compared to CON-fed calves. Evaluating the ^{13}C urea enrichment in plasma (Table 3, Supplemental Figure S2), we found that calves fed the SAL diet had a reduced ^{13}C urea turnover rate (12 %, 653 vs. 740 $\text{mg}/(\text{kg} \times \text{d})$, $P = 0.011$) and corresponding UER (61 vs. 70 g urea-N/d, $P = 0.060$) compared to calves receiving the CON diet. The urea pool size, recycling of urea-N into the gastrointestinal tract and urea hydrolysis rate did not

Table 2

Urea metabolism and rumen fluid analysis. $LSM \pm SE$.

Parameter ^a	SAL	CON	P -value ^b
^{13}C urea turnover, $\text{mg} \times \text{kg}^{-1} \text{ BW} \times \text{d}^{-1}$	653 $\pm$ 39	740 $\pm$ 28	0.011
Urea pool size, $\text{mg}/\text{kg BW}$	98 $\pm$ 5	113 $\pm$ 10	0.186
Urea-N pool size, g	9.2 $\pm$ 0.3	10.7 $\pm$ 1.2	0.181
UER, g urea-N/d	61 $\pm$ 2	70 $\pm$ 5	0.060
K_{urea} , h^{-1}	0.31 $\pm$ 0.01	0.29 $\pm$ 0.02	0.347
Urea space, L	138 $\pm$ 6	142 $\pm$ 8	0.519
GER, g urea N/d	47 $\pm$ 3	53 $\pm$ 4	0.282
GER/UER, %	79 $\pm$ 2	76 $\pm$ 1	0.274
UUN/UER, %	21 $\pm$ 2	24 $\pm$ 1	0.274
UHR, $\text{mmol}/30 \text{ min}$	27 $\pm$ 2	20 $\pm$ 3	0.176
Rumen fluid pH	6.8 $\pm$ 0.0	6.8 $\pm$ 0.1	0.979
Rumen fluid NH_3 , mM	2.6 $\pm$ 0.4	5.6 $\pm$ 0.4	0.003
Rumen fluid H_2 , μM^c	0.75 $\pm$ 0.46	0.22 $\pm$ 0.02	0.333

In weaned bull calves fed a diet containing willow leaves (SAL) or alfalfa hay (CON).

^a APE = atom percent excess; BW = body weight; GER = urea N transfer to the gastrointestinal tract; K_{urea} = fractional disappearance rate of ^{13}C urea; UER = urea-N entry rate; UHR = urea hydrolysis rate; UUN = urinary urea N excretion.

^b P -value from ANOVA analysis – diet effect. Further statistical data can be found in Supplemental Table S3.

^c $N = 12$; CON: $n = 5$; SAL: $n = 7$.

Table 3

Nitrogen balance and urinary N metabolites, benzoic acid and phenolic acid compounds. LSM $\pm$ SE.

Parameter ^a	SAL	CON	P-value ^b
N intake, g/d	105.8 $\pm$ 5.3	106.1 $\pm$ 6.7	0.961
N urine, g/d	25.3 $\pm$ 2.2	28.9 $\pm$ 1.9	0.278
N faeces, g/d	49.6 $\pm$ 2.8	40.7 $\pm$ 3.2	0.082
N balance	30.9 $\pm$ 2.3	36.5 $\pm$ 2.5	0.061
N use efficiency, %	29.5 $\pm$ 1.6	34.7 $\pm$ 1.0	0.050
AD of N, %	52.7 $\pm$ 1.5	61.4 $\pm$ 1.1	0.003
Urine volume, L/d	5.4 $\pm$ 0.4	5.9 $\pm$ 0.7	0.506
Urinary N metabolites, g/L			
Urea	5.7 $\pm$ 0.4	7.1 $\pm$ 0.7	0.065
Uric acid	0.30 $\pm$ 0.07	0.15 $\pm$ 0.02	0.114
Allantoin	2.33 $\pm$ 0.64	1.09 $\pm$ 0.29	0.092
Hippuric acid	10.1 $\pm$ 0.6	3.9 $\pm$ 0.7	0.002
Creatinine	0.88 $\pm$ 0.09	0.79 $\pm$ 0.07	0.495
Creatine	0.81 $\pm$ 0.13	0.78 $\pm$ 0.10	0.839
Phenolic acids (tannic acid-equ.), g/L	1.45 $\pm$ 0.04	0.80 $\pm$ 0.06	0.002
Salicylic acid, mg/L	53.9 $\pm$ 10.7	15.9 $\pm$ 5.4	< 0.001
Salicyluric acid, mg/L	101.4 $\pm$ 15.8	58.0 $\pm$ 17.0	0.190
Salicyl alcohol, mg/L	292.3 $\pm$ 12.0	219.75 $\pm$ 19.6	0.073
Benzoic acid, g/L	1.68 $\pm$ 0.61	2.10 $\pm$ 0.40	0.610

In weaned bull calves fed a diet containing willow leaves (SAL) or alfalfa hay (CON).

^a AD = apparent digestibility; Equ. = equivalent.

^b P-value from ANOVA analysis – diet effect. Further statistical data can be found in Supplemental Table S3.

differ between the dietary treatments (Table 3, Supplemental Figure S3). Ammonia in rumen fluid on the SAL diet was half that of the CON diet (2.6 vs. 5.6 mM, $P = 0.003$). *In vitro* H₂ production and pH of rumen fluid samples were not affected by dietary treatments.

3.3. Urine composition

The urinary N composition revealed that allantoin N (2.3 vs. 1.1, $P = 0.092$) and hippuric acid N (10.1 vs. 3.9 g/L, $P = 0.002$) concentrations were more than double in calves on the SAL diet compared to calves on the CON diet with comparable urine quantities (Table 3). Urinary hippuric acid accounted for 7.8 % and 17 % of urinary N excretions in CON-fed calves and SAL-fed calves, respectively. However, urinary urea N (5.7 vs. 7.1 g/L, $P = 0.065$) concentrations tended to be 20 % lower on the SAL diet than on the CON diet. Phenolic acid concentrations in urine were 81 % higher on the SAL diet than on the CON diet ($P = 0.002$). Although urinary benzoic acid and salicyluric acid concentrations did not differ between diets, SAL-fed calves excreted twice as much salicylic acid (54 vs. 16 mg/L, $P < 0.001$) and tended to excrete 33 % more salicyl alcohol (292 vs. 220 mg/L, $P = 0.073$) with urine than CON-fed calves.

3.4. N₂O and NH₃ emissions and isotopic signatures of soil incubation

The incubation of soil with cattle urine revealed a 14 % lower NH₃-N emission rate and a 16 ‰ reduction in $\delta^{15}\text{N}_{\text{NH}_3}$ with the use of urine from SAL- than CON-fed calves (38.4 vs. 44.9 $\mu\text{g}/(\text{h} \times \text{kg soil})$, $P < 0.01$) (Table 4). Although the reduction in the NH₃-N emission rate was more pronounced during the first 24 h of incubation, differences in $\delta^{15}\text{N}_{\text{NH}_3}$ were predominantly found between 6 and 48 h of incubation (Fig. 1A, Supplemental Figure S4). Differences in the average NH₃-N emission rates disappeared when related to the urinary urea N concentration (Table 4). The N₂O-N record showed that soil incubated with urine from SAL-fed calves released 96 % less N₂O-N between 24 and 48 h of incubation, when potentially high N₂O-N emissions occurred, than soil incubated with urine from CON-fed calves (0.11 vs. 2.69 $\mu\text{g}/(\text{h} \times \text{kg soil})$, $P < 0.001$) (Fig. 1B). The average N₂O-N emission rate was 81 % lower for soil incubated with SAL than with CON-derived urine (0.34 vs.

1.75 $\mu\text{g}/(\text{h} \times \text{kg soil})$, $P < 0.001$) and 83 % diminished when related to the urinary N content (0.001 % vs. 0.006 %, $P < 0.001$) (Table 4). We found no differences in soil NH₄⁺ or its isotopic signature (Table 4, Supplemental Table S4). However, the soil NO₃⁻ content tended to be 36 % elevated when incubated with urine from CON-fed than SAL-fed calves ($P < 0.1$), regardless of the urinary N concentration. Scaling these findings to manure left on pastures by non-dairy cattle and to countries with comparable climatic conditions and willow species revealed that the provision of willow leaves to cattle on pasture could mitigate 50 kt of urine-derived NH₃-N (14 %) and 89 kt of urine-derived N₂O-N (81 %) emissions per year compared to 2021 (Fig. 2A and B).

The incubation experiment with artificial urine revealed an 11-fold higher NH₃-N emission rate from AU with hippuric acid than without (49.8 vs. 4.3 $\mu\text{g}/(\text{h} \times \text{kg soil})$, $P < 0.001$), but this increase tended to be reduced by 10 % when salicylates were added (49.8 vs. 44.6 $\mu\text{g}/(\text{h} \times \text{kg soil})$, $P < 0.1$) (Table 4). In contrast, the average NH₃-N emission rate was reduced by 42 % with the addition of salicylic acid compared to AU alone (2.5 vs. 4.3 $\mu\text{g}/(\text{h} \times \text{kg soil})$, $P < 0.05$), especially between 24 and 48 h of incubation (Fig. 1C). The addition of salicyluric acid, salicylates and hippuric acid depleted the average $\delta^{15}\text{N}_{\text{NH}_3}$ by 17, 16 and 21 ‰, respectively, compared to AU without additives ($P < 0.01$) (Supplemental Table S4). Mixing AU with hippuric acid and salicylates further reduced $\delta^{15}\text{N}_{\text{NH}_3}$ by 5 ‰ towards AU with hippuric acid alone ($P < 0.05$). The addition of hippuric acid caused an 88 % mitigation of the average N₂O-N emission rate compared to AU alone (0.46 vs. 4.36 $\mu\text{g}/(\text{h} \times \text{kg soil})$, $P < 0.05$) (Table 4). This reduction was even more pronounced when considering measurements above or under the detection limits (Fig. 1D). Moreover, the addition of hippuric acid was associated with an $\delta^{15}\text{N}_{\text{N}_2\text{O}}$ isotopic signature depleted by 13 ‰ ($P < 0.001$), on average, but $\delta^{15}\text{N}_{\text{N}_2\text{O}}^{\text{bulk}}$ was elevated 53 ‰, on average ($P < 0.01$) (Supplemental Table S4, Supplemental Figure S4). The addition of salicylates caused an elevation of N₂O-N emission rates by 84 % ($P < 0.05$, Table 4), particularly between 24 and 48 h of incubation (Fig. 1D). This elevation was associated with an 6 ‰ enrichment in $\delta^{18}\text{O}_{\text{N}_2\text{O}}$ ($P < 0.05$) (Supplemental Table S4). Furthermore, soil incubated with AU mixed with hippuric acid had more than double the amount of NH₄⁺ (114 vs. 41 mg N $\times$ kg⁻¹ soil) and NO₃⁻ (12 vs. 5 mg N $\times$ kg⁻¹ soil) than soil incubated with AU alone ($P < 0.01$) (Table 4). At the same time, a 2.8 ‰ depletion in $\delta^{15/14}\text{N}_{\text{NH}_4^+}$ ($P < 0.1$) and a 15.6 ‰ enrichment in $\delta^{15/14}\text{N}_{\text{NO}_3^-}$ ($P < 0.05$) occurred in soil incubated with hippuric acid compared to that without (Supplemental Table S4).

The estimated fractional contributions of individual pathways involved in N₂O release (Supplemental Figure S5) revealed that the relative contribution of bD to N₂O release was 29.3 % lower after incubation with AU and hippuric acid than with AU alone (35.6 % vs. 64.9 %). In contrast, the fD and Ni fractions contributed 27.1 % (40.9 % vs. 13.8 %) and 5.5 % (12.3 % vs. 6.8 %) more to N₂O release, respectively, from AU mixed with hippuric acid than that without. However, the absolute partitioning of individual pathways involved in N₂O release revealed an overall reduced contribution of bD (2.8 vs. 0.2 $\mu\text{g}/(\text{h} \times \text{kg soil})$), nD (0.6 vs. 0.1 $\mu\text{g}/(\text{h} \times \text{kg soil})$), fD (0.6 vs. 0.2 $\mu\text{g}/(\text{h} \times \text{kg soil})$) and Ni (0.3 vs. 0.1 $\mu\text{g}/(\text{h} \times \text{kg soil})$) when AU was mixed with hippuric acid (Table 4). The residual unreduced N₂O-N fraction was 20.3 % higher (27.9 % vs. 7.6 %, Supplemental Figure S5), whereas the resulting N₂-N releases were lower (1.2 vs. 53.0 $\mu\text{g}/(\text{h} \times \text{kg soil})$, Table 4) in soils incubated with AU mixed with hippuric acid compared to AU alone. When AU was mixed with salicylates, the relative contribution of bD to N₂O release decreased by 25.8 % (39.1 % vs. 64.9 %), whereas the contribution of fD and Ni increased by 22.0 % (13.8 % vs. 35.8 %) and 5.3 % (6.8 % vs. 12.1 %), respectively. However, the absolute contribution of fD (0.6 vs. 2.9 $\mu\text{g}/(\text{h} \times \text{kg soil})$) and Ni (0.3 vs. 0.9 $\mu\text{g}/(\text{h} \times \text{kg soil})$) increased 5 and 3 times, respectively, compared to AU, whereas the contributions of bD and nD were comparable. The residual N₂O fraction was 28 % (38.9 % vs. 11.4 %) lower and N₂-N release 3 times higher (10.7 vs. 62.4 $\mu\text{g}/(\text{h} \times \text{kg soil})$) of AU mixed with

Table 4Average urinary emissions (N_2O , NH_3 and N_2), soil composition and processes involved in N_2O release.^a

Parameter	SAL	CON		Artificial urine ^b	Artificial urine +								HA + SD	HA vs. HA + SD
					Salicylic acid	Salicyluric acid	Salicyl alcohol	Salicylates (SD)	Hippuric acid (HA)					
$\text{NH}_3\text{-N}$, $\mu\text{g} \times \text{h}^{-1} \times \text{kg}^{-1}$ soil	38.4	44.9	* *	4.3	2.5	*	4.4	3.5	3.4		49.8	* **	44.6	* ** #
$\text{N}_2\text{O-N}$, $\mu\text{g} \times \text{h}^{-1} \times \text{kg}^{-1}$ soil	0.34	1.75	* **	4.36	6.53		8.65	5.50	8.03	*	0.46	*	0.62	* *
$\text{NH}_3\text{-N}$, % of urine-N $\times \text{d}^{-1}$	0.13	0.15	* *	0.02	0.01	*	0.02	0.01	0.01		0.16	* *	0.14	* ** #
$\text{NH}_3\text{-N}$, % of urine-urea-N $\times \text{d}^{-1}$	0.24	0.23		0.02	0.01	*	0.02	0.02	0.02		0.24	* **	0.22	* ** #
$\text{N}_2\text{O-N}$, % of urine-N $\times \text{d}^{-1}$	0.001	0.006	* **	0.016	0.024		0.032	0.021	0.030	*	0.002	*	0.002	* *
$\text{NH}_4\text{-N}$, mg N $\times \text{kg}^{-1}$ soil	153	156		41.1	34.1		43.3	41.2	42.3		114.2	* *	105.7	* *
$\text{NO}_3\text{-N}$, mg N $\times \text{kg}^{-1}$ soil	19.5	26.7	#	4.8	4.5		4.8	5.9	5.1		11.9	* *	9.6	* **
$\text{NH}_4\text{-N}$ soil, % of urine-N	22.4	22.3		6.4	5.3		6.8	6.4	6.6		15.0	* *	13.9	* **
$\text{NO}_3\text{-N}$ soil, % of urine-N	2.8	3.8	#	0.8	0.7		0.8	0.9	0.8		1.6	* *	1.3	* **
$\text{N}_2\text{-N}$, $\mu\text{g} \times \text{h}^{-1} \times \text{kg}^{-1}$ soil ^{FR}	n.m.	n.m.		53.0	10.2	-	10.7	-	62.4	-	1.2	-	1.9	- -
$\text{N}_2\text{-N}$, % of urine-N $\times \text{d}^{-1\text{FR}}$	n.m.	n.m.		0.195	0.038	-	0.040	-	0.233	-	0.005	-	0.006	- -
Absolute partitioning of sources involved in $\text{N}_2\text{O-N}$ release, $\mu\text{g} \times \text{h}^{-1} \times \text{kg}^{-1}$ soil ^c														
Bacterial denitrification	n.m.	n.m.		2.83	2.08	-	2.56	-	1.88	-	3.14	-	0.16	- -
Nitrifier denitrification	n.m.	n.m.		0.63	0.98	-	1.62	-	0.71	-	1.04	-	0.07	- -
Fungal denitrification	n.m.	n.m.		0.60	2.44	-	2.86	-	2.18	-	2.88	-	0.08	- -
Nitrification	n.m.	n.m.		0.30	1.03	-	1.61	-	0.73	-	0.92	-	0.15	- -

Statistical analyses (*t*-test) between pooled urine samples of weaned bull calves fed a diet containing willow leaves (SAL) or alfalfa hay (CON), between artificial urine (AU) and AU with additives and between AU with hippuric acid and hippuric acid with salicylates (150 g urine / kg soil, each $n = 5$). * ** = $P < 0.001$, * = $P < 0.01$, * = $P < 0.05$, # = $P < 0.1$, - = no statistical analysis. n.m. = not measured.

^{FR} based on the residual unreduced $\text{N}_2\text{O-N}$ fraction determined in **FRAME** (Lewicki et al., 2022) (Supplemental Figure S5).

^a Standard errors and further statistical data can be found in Supplemental Table S3.

^b If not specified differently $n = 5$ per approach and time point. N_2O : 1 h incubation – AU + Salicylates $n = 4$; 3 h incubation – AU $n = 4$, AU + Salicylic acid $n = 2$, AU + Salicylic alcohol $n = 4$, AU + Salicylates $n = 4$, AU + Hippuric acid $n = 3$, AU + Hippuric acid + Salicylates $n = 4$; 6 h incubation – AU + Salicylic acid $n = 2$, AU + Salicyluric acid $n = 4$, AU + Salicylic alcohol $n = 4$, AU + Salicylates $n = 4$, AU + Hippuric acid $n = 3$, AU + Hippuric acid + Salicylates $n = 4$; 24 h incubation – AU $n = 2$, AU + Salicylic acid $n = 1$, AU + Salicyluric acid $n = 1$, AU + Salicylic alcohol $n = 2$, AU + Salicylates $n = 3$, AU + Hippuric acid $n = 4$, AU + Hippuric acid + Salicylates $n = 4$; 48 h incubation - AU + Salicylic acid $n = 4$, AU + Salicyluric acid $n = 4$, AU + Hippuric acid $n = 3$, AU + Hippuric acid + Salicylates $n = 3$.

^c Calculated from $\text{N}_2\text{O-N}$ release and the relative partitioning of sources (Supplemental Figure S5).

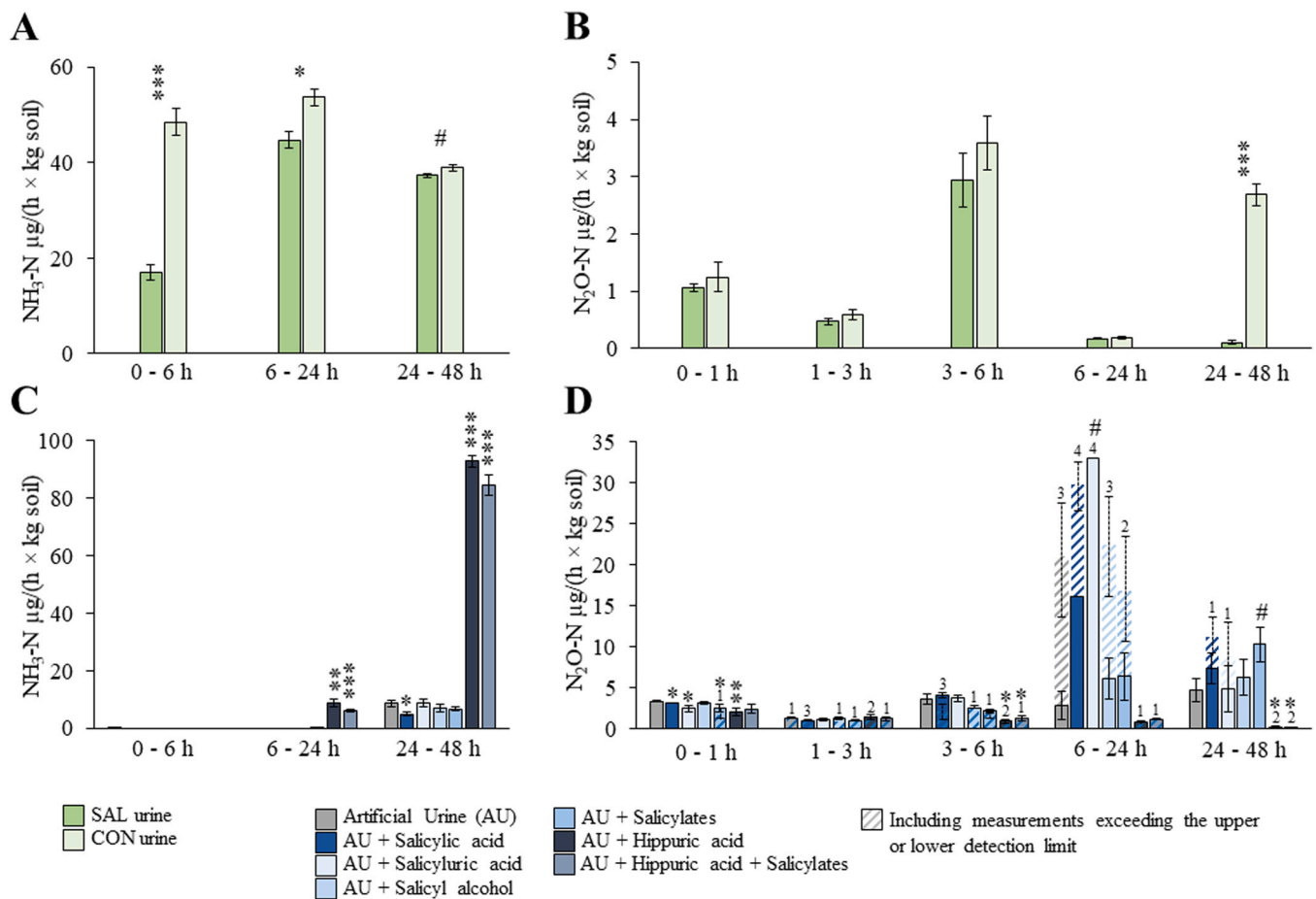


Fig. 1. Time course of ammonia-N ($\text{NH}_3\text{-N}$) and nitrous oxide-N ($\text{N}_2\text{O-N}$) emission rates for 48 h. Different urine compositions were applied on soil (150 g urine / kg soil, each $n = 5$) for 48 h. Urine derived from weaned bull calves fed a diet containing willow leaves (SAL) or alfalfa hay (CON) (A and B). Artificial urine (AU) was prepared according to the average urine nitrogen composition from animals fed the CON diet: 7.08 g/L urea, 0.15 g/L uric acid, 1.09 g/L allantoin, 0.79 g/L creatinine and 0.78 g/L creatine in distilled water. In the AU, 53.94 mg/L salicylic acid, 101.36 mg/L salicyluric acid, 292.32 mg/L salicyl alcohol, or 10.08 g/L hippuric acid were dissolved to achieve the mean concentration as the urine from animals fed the SAL diet (C and D). Statistical analyses (two-sample t -test) between SAL and CON and between AU and AU with additives were done for each time point of sampling separately. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$, # = $P < 0.1$. Data are given as mean $\pm$ SEM (Supplemental Table S3). Shading in D – the number of samples reaching the upper or lower detection limit (0.11 and 88.75 ppm) were given (out of $n = 5$) and the potential mean $\pm$ SEM was plotted, if these measurements would be considered.

salicylates than of AU mixed with salicylic acid, salicyluric acid, or salicyl alcohol. There were no notable differences in the fractional contributions of individual pathways to $\text{N}_2\text{O-N}$ release among vessels incubated with AU mixed with additives (Supplemental Figure S5).

4. Discussion

Willow leaves have a decent nutritional value for ruminants, but they are also rich in bioactive compounds such as phenols, salicylates and tannins, all of which possess a high potential to diminish CH_4 and urinary N emissions. The concentration, composition, type and structure of these compounds strongly depend on the environmental growth conditions of the fodder tree (Luske and van Eekeren, 2018) and preservation method. As such, it is not surprising that total phenolic compounds, tannin and salicylate concentrations of willow leaves were slightly higher than those reported in previous studies (McWilliam et al., 2005; Muklada et al., 2018).

Tannin intake may inhibit ruminal fermentation processes because tannins interact with fibrous carbohydrates to form carbohydrate complexes, which hinder the fermentation of fibre and reduce H_2 delivery for methanogenesis (Jayanegara et al., 2012). However, tannins may also directly suppress the viability of methanogens (Jayanegara et al., 2012). In the present study, we found no differences in CH_4 production

per kg aNDF intake, total CH_4 emission, or rumen fluid H_2 concentration between SAL- and CON-fed calves, suggesting no major impacts of willow tannins on ruminal fermentation of carbohydrates. This conclusion is supported by the comparable DM and aNDF digestibility of both diets. However, we found an 8 % lower CH_4 emission /kg metabolic body weight in SAL-fed calves. Comparably, Ramirez-Restrepo et al. (2010) observed a 20 % reduction in CH_4 emission/kg metabolic body weight in sheep supplemented with willow leaves containing 12 g tannins per kg DM compared to sheep fed only on pasture, and the organic matter digestibility was not affected compared to the control. Based on meta-analysis, Jayanegara et al. (2012) concluded that the variation in CH_4 production was very high when tannin concentrations were ~ 20 g/kg DM intake, whereas the variability clearly decreased with higher tannin concentrations. The low levels of willow tannins used in the present and previous studies (Ramirez-Restrepo et al., 2010) may not have been sufficient to reduce the total CH_4 emission, and other dietary components may have masked the effect of low tannin concentrations.

In addition to their interaction with structural carbohydrates, tannins bind to dietary proteins via hydrogen bonds, thereby forming complexes that inhibit the proteolytic and ureolytic activity of rumen microbes (Herremans et al., 2020). Min et al. (2003) postulated that the formation of these protein complexes was pH-reversible; thus, the complexes would dissociate under the low pH conditions of the

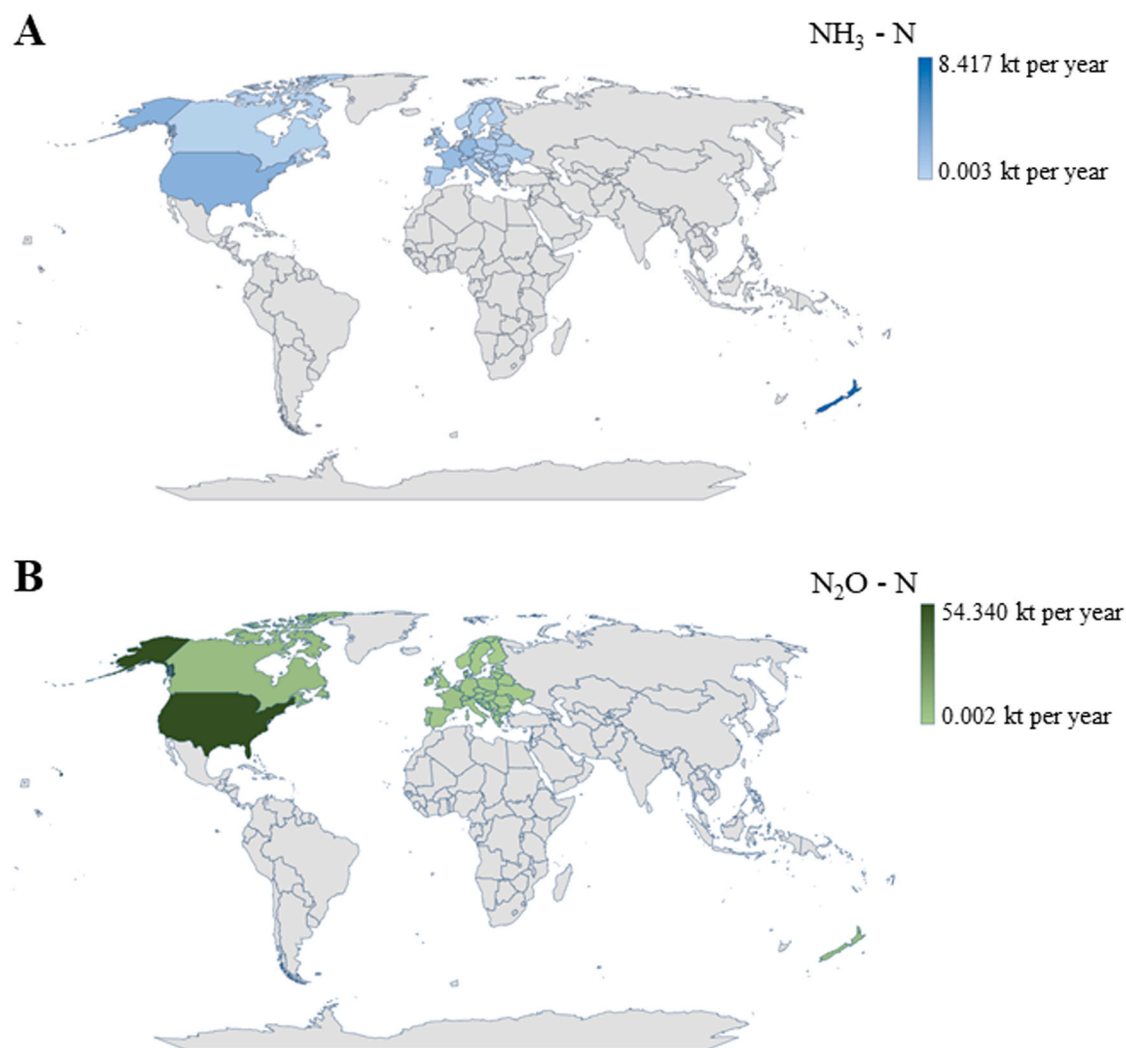


Fig. 2. Upscaling of the urinary ammonia ($\text{NH}_3\text{-N}$, **A**) and nitrous oxide ($\text{N}_2\text{O-N}$, **B**) emission mitigation potential. Ammonia and nitrous oxide emissions were upscaled relative to 2021, when willow leaves would be supplemented to non-dairy cattle on pasture. Data are calculated as absolute mitigation potential per year and country (FAO, 2023). Data on N_2O emissions and the content of N derived from manure left on pasture by non-dairy cattle in the year 2021 were considered in countries with comparable climatic conditions and willow species (Argus et al., 2010; Durrant et al., 2016). Approximately 91.1 % of the $\text{N}_2\text{O-N}$ emissions from cattle manure left on pasture derived from urine patches (Voglmeier et al., 2019). Total N volatilised as NH_3 accounts for 22.4 % of N excreted by cattle on pasture and urine patches account for 88.6 % of these NH_3 emissions (Laubach et al., 2013).

abomasum. Therefore, low tannin concentrations (20–45 g/kg DM) may inhibit protein degradation in the rumen and thus reduce the availability of amino acids in the small intestine (Min et al., 2003). A meta-analysis by Herremans et al. (2020) revealed that the inclusion of 9.5 g tannins per kg DM inhibited ruminal proteolysis, as observed the 16 % reduction in rumen NH_3 concentration. We confirmed this finding, showing that the intake of ~26 g tannins per kg DM reduced ruminal NH_3 concentrations by 55 %. Dietary supplementation with salicylic acid inhibits bacterial urease activity (Mao et al., 2009) and modulates ruminal proteolysis (Kingston-Smith et al., 2012). However, willow leaves do not contain free salicylic acid, which raises the question of whether salicylic acid precursors, such as tremulacin, salicin and salicortin, found in willow leaves affect ruminal proteolytic processes.

Despite the differences in ruminal NH_3 concentrations, plasma and urine concentrations of allantoin and uric acid summed as purine derivatives were comparable between the SAL- and CON-fed groups, indicating a comparable level of rumen microbial protein synthesis (Tas and Susenbeth, 2007). In line with this, tannin inclusion of up to 23 g/kg DM in the ration of cattle did not affect microbial protein yield (Tseu et al., 2020). With comparable microbial protein synthesis, the SAL-fed animals dispensed with 8.7 % of the N digestibility to achieve the same

body condition and growth rate as the CON-fed animals. Comparably, Herremans et al. (2020) showed that dietary tannins reduced the N digestibility in dairy cows by 4 % but did not diminish milk production. Our and previous (Herremans et al., 2020) results suggest no long-term negative effects on animal productivity of cattle fed willow. However, the effects on the productivity of ruminants other than cattle, particularly of browsing and intermediate small ruminants, may be different from grazers and need to be investigated in future studies.

The reduced ruminal NH_3 concentrations during willow feeding likely resulted in less NH_3 absorption and reduced hepatic urea synthesis in SAL-fed calves. This is reflected by a decline in metabolic urea turnover and reduced plasma and urinary urea concentrations in calves fed the SAL diet compared to the CON diet. However, the urea transfer rate to the gastrointestinal tract, rumino-hepatic urea-N recycling and the urea hydrolysis rate did not differ between the SAL- and CON-fed groups, indicating that comparable amounts of urea were used for microbial protein synthesis. These results agree with an earlier study in sheep demonstrating that 7.7 g/kg DM of tannins from *Acacia* extract did not affect the urea flux across the portal drained viscera, liver and splanchnic tissues but decreased the urinary excretion of urea (Orlandi et al., 2020).

The SAL-fed calves tended to excrete 20 % less urinary urea but 22 % more faecal N than the CON-fed calves. The shift from urinary urea N to faecal N excretion has been previously reported (Terranova et al., 2021) and is associated with a reduction in volatile N emissions because urinary N excretions account for 91 % of N_2O and 87 % of NH_3 emissions from manure (Laubach et al., 2013; Voglmeier et al., 2019). In addition, SAL-fed calves excreted 2.6-fold more urinary hippuric acid and had greater plasma hippuric acid concentrations than their counterparts. Because hippuric acid is formed by the conjugation of glycine with benzoic acid in the liver and benzoic acid is not synthesised by mammalian metabolism, willow leaves must be a source of metabolically available benzoic acid. However, there were no detectable amounts of benzoic acid in the willow leaves. Instead, the phenolic acid compounds 4-hydroxycinnamic acid and ferulic acid, both flavonoid precursors and abundantly present in willow leaves (Tawfeek et al., 2021), are ruminally degraded to benzoic acid and a source of hippuric acid (Martin, 1982). Furthermore, feeding 26 g tannic acid per kg DM intake of cattle increased urinary hippuric acid excretion by 7 % compared to non-supplemented counterparts (Yang et al., 2016).

The average hippuric acid output in beef cattle urine ranges from 2.2 % to 7.3 % of urinary N excretion, when the diet is free of tannins (Gao and Zhao, 2022). In the present study, urinary hippuric acid excretion accounted for 7.8 % of the urinary N excretion in the CON-fed group and for more than 17 % in SAL-fed animals. The great increase in urinary hippuric acid N excretion exceeded the decrease in urinary urea N excretion in SAL calves and thus prevented the reduction in total urinary N excretions that is usually observed when feeding tannins (Terranova et al., 2021) or tannic acid (Yang et al., 2016). Urinary urea, the primary source of urine-derived NH_3 emissions, and hippuric acid increase the urea hydrolysis rate when applied to soil, indicating that hippuric acid is a driver of these NH_3 emissions (Whitehead et al., 1989). In soil, hippuric acid is cleaved to benzoic acid and glycine; the latter is further deaminated to ammonium, increasing soil NH_3 release (Whitehead et al., 1989). Interestingly, differences in urine-derived NH_3 emissions from SAL- and CON-fed animals were pronounced at the beginning but converged during the course of incubation. The lower NH_3 emission rate during the first 6 h of incubation (Fig. 1A) can be explained by the lower urea concentration in the urine of SAL-fed calves. After 6 h of incubation, the effect of hippuric acid on NH_3 formation increased (Fig. 1C), leading to the convergence of NH_3 emissions between 6 and 24 h (Fig. 1A). Although the soil incubation experiments with cattle urine and artificial urine were conducted separately and thus revealed slight differences in NH_3 and N_2O emission kinetics, we identified a mitigating effect of salicylic acid on NH_3 emissions from artificial urine, indicating that the higher salicylic acid concentration excreted in urine reduces urine-derived NH_3 emissions from SAL-fed calves. One underlying mechanism is the binding of salicylic acid to urea, preventing urea hydrolysis (Silva et al., 2020), whereas it does not affect soil urease activity (Holik et al., 2016). To our knowledge, this is the first study demonstrating that, despite the higher urinary hippuric acid concentrations, the lower urinary urea and presence of salicylic acid mitigate urine-derived NH_3 emissions in SAL compared to CON calves.

Previous studies (van Groenigen et al., 2006; Bertram et al., 2009) described hippuric acid as a natural inhibitor of N_2O emissions released from urine patches. Both hippuric acid and its breakdown product benzoic acid have been shown to inhibit denitrification processes in soil and potentially also nitrification processes (Bertram et al., 2009). The strong mitigating effect of hippuric acid on N_2O and N_2 found after 3 h of incubation with AU occurred in parallel to changes in $\delta^{15}\text{N}_{\text{N}_2\text{O}}^{\text{bulk}}$ and $\delta^{15}\text{N}_{\text{N}_2\text{O}}^{\text{SP}}$. The latter indicates a percentage shift from bD and nD processes towards the contribution of dissimilatory nitrate reduction to ammonium (DNRA) (Rohe et al., 2016), which is consistent with the reduced N_2O but increased NH_3 emissions. Interestingly, the comparison of AU and AU mixed with hippuric acid revealed a 90 % reduction of N_2O release. Our results revealed that both the absolute denitrification and nitrification processes involved in N_2O release appeared to be

inhibited by the addition of hippuric acid, but this inhibition was much more pronounced for bD and nD than for fD and Ni. Consequently, we attributed the accumulation of NO_3^- and NH_4^+ in soils incubated with hippuric acid-containing AU at least partly to an absolute decline in denitrification and nitrification processes involved in N_2O release, an increase in DNRA and the deamination of glycine to NH_4^+ (Whitehead et al., 1989).

In line with the addition of hippuric acid, FRAME analysis revealed that the addition of salicylates shifted the relative contribution of bD towards the relative contribution of fD and Ni to N_2O release. However, N_2O release was almost doubled by the addition of salicylates, but this increase in N_2O was much less pronounced compared to the mitigating effect of hippuric acid on N_2O . Salicylic acid has been shown to enhance the enzymatic rates of nitrate reductases in wheat seedlings (Hayat et al., 2005) and strongly increase N_2O emissions from soil (Giles et al., 2012). Therefore, it can be concluded that the N_2O emissions derived from the urine of calves supplemented with willow leaves were strongly reduced by the effect of hippuric acid, which masked the effect of salicylates to increase N_2O emissions.

The dominant role of hippuric acid in reducing N_2O emissions from urine indicated an increasing concentration of NO_3^- and NH_4^+ in soils after incubation with urine from SAL-fed calves compared to CON-fed calves. Surprisingly, despite the lower volatile N losses from the urine of SAL-fed calves, the soil NH_4^+ concentration did not differ from the CON-fed group, and the soil NO_3^- concentration was slightly decreased when soil was incubated with urine from SAL-fed compared to CON-fed calves. The latter suggests the involvement of urinary compounds other than those specifically tested in the present study, which may diminish the mineralisation processes and thus the replenishment of soil NH_4^+ and NO_3^- . Phenolic acids and tannins lower the accumulation of NO_2^- , a key precursor of N_2O and NO_3^- , by inhibiting mineralisation and nitrification rates in soil (Clemensen et al., 2020). In addition, Chen et al. (2020) demonstrated that phenolic acids negatively correlated with N mineralisation rates in soil *ex situ* and that some of these phenolic acids were found in willow leaves (Tawfeek et al., 2021). Adamczyk et al. (2011) reported that tannins positively correlated with the amount of precipitated organic N in soils, suggesting that tannins inhibit soil N mineralisation and increase humification. Consequently, the application of urine from willow-fed cattle on pasture may prevent rapid N mineralisation and thus might be an important strategy to regulate soil NO_3^- , reduce NO_3^- leaching and eventually ameliorate long-term humification by preventing early N mineralisation (Horner et al., 1988; Clemensen et al., 2020).

5. Conclusions

The combination of *in vivo* and *ex vivo* studies, partially conducted with the help of stable isotope tracer techniques herein, provided the first insights into how supplementation of cattle nutrition with willow leaves reduced the environmental N load, involving the reduction in N digestibility, urea metabolism and urinary urea excretion of cattle, urine-derived NH_3 and N_2O emissions and soil NO_3^- formation. We found that despite increased urinary hippuric acid concentrations, elevated salicylic acid and reduced urea concentrations mitigated urinary NH_3 emissions, as hypothesised. Urinary salicylates alone increased urine-derived N_2O emissions, but the increased urinary hippuric acid concentration in willow-fed cattle masked the effect of salicylates, leading to a net mitigation of urine-derived N_2O emissions. This effect was mainly attributed to the inhibition of bacterial denitrification processes involved in N_2O release from soil. Overall, the supplementation of cattle nutrition with willow leaves resulted in a 14 % mitigation of urinary NH_3 emissions and an 81 % mitigation of urinary N_2O emissions. Extrapolating these results to a global scale shows that the provision of willow leaves to cattle on pasture may mitigate 50 kt of urine-derived $\text{NH}_3\text{-N}$ and 89 kt of urine-derived $\text{N}_2\text{O-N}$ emissions per year. However, the mitigation potentials of NH_3 and N_2O emissions strongly depended

on the presence of comparable willow species as fed in the present study and on the number of non-dairy cattle on pasture. The reduction of soil NO_3^- formation after incubation with urine from willow-fed calves suggests an inhibition of N mineralisation processes, but further research is needed to determine if urine from willow-fed cattle also reduces NO_3^- leaching and facilitates humification. To continue decreasing the environmental N footprint from cattle urine, the potential effects of further plant secondary metabolites on mitigating N emissions should be considered and our results should be further tested under different grazing conditions in the field.

Ethics approval and consent to participate

All experimental procedures on animals were approved by the Office of Agriculture, Food Security and Fishery Mecklenburg-Western Pomerania, Germany (No. 7221.3-1-004/22).

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CRediT authorship contribution statement

Kuhla Björn: Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Müller-Kiedrowski Carolin:** Visualization, Investigation, Formal analysis, Data curation. **Görs Solvig:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Mittermeier-Kleßinger Verena:** Writing – review & editing, Software, Resources, Methodology, Formal analysis. **Dawid Corinna:** Writing – review & editing, Software, Resources, Methodology. **Wrage-Mönnig Nicole:** Writing – review & editing, Validation, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions

CBMM-K conducted the animal experiment, wrote the original draft and created the visualizations, BK, VM-K and NW-M conducted reviews and editing of the original draft, BK and CBMM-K conceptualised the study design and paper, SG, VM-K, CD, CBMM-K and NW-M contributed to the methodology and data investigation.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.agee.2025.109671](https://doi.org/10.1016/j.agee.2025.109671).

Data availability

Data will be made available on request.

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Glossary

- AD: apparent digestibility
 ADF: acid detergent fibre
 ADL: acid detergent lignin
 aNDF: amylase-treated neutral detergent fibre
 AU: artificial urine
 bD: bacterial denitrification
 BW: body weight
 C_{diH2}: hydrogen concentration in the sieved rumen fluid
 C_{gH2}: hydrogen concentration in the gaseous phase
 CO₂: carbon dioxide
 CH₄: methane
 CON: control group
 δ¹⁵N_{N2O}^{bulk}: isotope ratio of ¹⁵N and ¹⁴N in N₂O corrected against a standard
 δ¹⁵N_{N2O}^{SP}: site preference of ¹⁵N towards the α or β N position in N₂O
 δ¹⁸O_{N2O}: isotope ratio of ¹⁸O and ¹⁶O in N₂O corrected against a standard
 DM: dry matter
 DNRA: dissimilatory nitrate reduction to ammonium
 fD: fungal denitrification
 FRAME: Fractionation and Mixing Evaluation
 GER: urea entrance rate into the gastrointestinal tract
 H₂: hydrogen
 HP: Heat production
 IRMS: isotope ratio mass spectrometer
 mBW: metabolic BW
 K_{urea}: fractional disappearance rate of ¹³C urea
 N: nitrogen
 Ni: nitrification
 nD: nitrifier denitrification
 NH₃: ammonia
 NH₄⁺: ammonium
 N₂O: nitrous oxide
 NO₂: nitrite
 NO₃⁻: nitrate
 N_{urine}: urinary N excretion

O : oxygen	UER : urea entry rate
Q : urea pool size a	V_g : volume of the gas phase
r_{N_2O} : mean residual unreduced N_2O -N fraction	V_{rf} : volume of rumen fluid
SAL : group supplemented with willow leaves (<i>Salix spp.</i>)	